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ATTORNEY'S DOCKET NUMBER: 1051-1-020

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INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA00/00483	April 27, 2000	April 28, 1999

TITLE OF INVENTION

TELOPHASE ENUCLEATED OOCYTES FOR NUCLEAR TRANSFER

APPLICANT(S) FOR DO/EO/US

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [X] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is attached hereto (required only if not communicated by the International Bureau).
   b. [X] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [X] is attached hereto.
   b. [X] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [X] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [X] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [X] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). UNEXECUTED
10. [X] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information: One (1) Sheet of Drawings (attached to Published Application); Preliminary Examination Report (which includes the amended claims)

EXPRESS MAIL CERTIFICATE NO.: EL 920250815 US DATE OF DEPOSIT: OCTOBER 26, 2001

531 Rec'd PC: 26 OCT 2001

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/018375	PCT/CA00/00483	1051-1-020

21. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	38 - 20 =	18	x **$18.00**	$ 324.00
Independent claims	7 - 3 =	4	x **$84.00**	$ 336.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$
TOTAL OF ABOVE CALCULATIONS =				$ 1,550.00
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			+	$ 775.00
SUBTOTAL =				$ 775.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$
TOTAL NATIONAL FEE =				$ 775.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$
TOTAL FEES ENCLOSED =				$ 775.00
			Amount to be refunded:	$
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a. ☒ A check in the amount of $ 775.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

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EXPRESS MAIL CERTIFICATE NO.:EL920250815 US DATE OF DEPOSIT:OCTOBER 26, 2001

TELOPHASE ENUCLEATED OOCYTES FOR NUCLEAR TRANSFER

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The present invention relates to an improved method for obtaining an enucleated host oocyte for transferring nuclei from embryonic, germinal and somatic cells with the objective of cloning or multiplying mammals, and to a method of reconstituting an animal embryo.

(b) Description of Prior Art

The technique of nuclear transfer has been widely used to multiply embryos by transferring blastomere nuclei from early-stage embryos into enucleated oocytes. This technique enables an increase in the yield of embryos produced from parents of top genetic value, enabling to accelerate the annual genetic gain within an animal population. Nuclear transfer has also been used with nuclei from cell lines derived from embryonic (Campbell et al., 1996, *Nature* **380**:64-66), fetal and adult tissue (Wilmut et al., 1997, *Nature* **385**:810-813). By using nuclei from an unlimited source, nuclear transfer from cell lines enables not only the production of a larger number of genetically identical offspring but also an opportunity for modifying the genetic characteristic of cells *in vitro* prior to the production of live offspring, enabling the production of transgenic mammals. Moreover, the use of cells from adult animals for nuclear transfer, either directly or through previous *in vitro* passage, enable the multiplication (cloning) of animals of known phenotypes.

Basically, the nuclear transfer technique requires a donor nucleus to provide the genetic material of choice and a host oocyte to provide the cytoplasm that plays a role in reprogramming the

nucleus to support embryo development. With the nuclear and cytoplasm sources in hand, three main steps are required to reconstruct an oocyte by nuclear transfer. First, host oocytes need to be enucleated to remove all nuclear genetic material. This step is usually performed by microsurgical removal of the chromosomes from either a metaphase plate or pronuclei. Second, donor nuclei need to be introduced into the oocyte (nuclear transfer). This step is normally obtained by fusing the membranes of the nuclear donor cell and the host oocyte. However, nuclear transfer can also be obtained by traversing the oocytes plasma membrane and microinjecting the nucleus directly into the host cytoplasm. Finally, non-activated host oocytes need awakening from their meiotic arrest (oocyte activation). This step can be achieved by exposing the oocyte to a physical stimulus, such as temperature changes or an electric shock, or exposing the oocyte to chemical agents, such as ethanol or exogenous calcium. The order in performing each of the steps above can vary in different situations and may have an important effect on the ability of the reconstructed oocyte to undergo further development.

In mice, oocyte enucleation was performed after fertilization by visualizing and removing the pronuclei by microsurgery. This enucleation technique is less efficient in other mammals due to the higher density of cytoplasm resulting in poor visualization of pronuclei. Moreover, attempts to use pronuclear-stage enucleated oocytes led invariably to poor developmental rates when using cleavage stage blastomeres as nuclear donors. Improved development after nuclear transfer was achieved initially in sheep (Willadsen,S. 1986, *Nature* **320**:63-65) and later in other mammals using host oocytes that had not been activated at the time of

fusion. However, a problem remained that metaphase stage chromatin cannot be visualized easily by microscopy in most mammals. Willadsen (Willadsen, S.1986, *Nature* **320**:63-65) proposed an enucleation procedure in which sheep oocytes were blindly divided into halves either containing or not the first polarbody. To avoid a large loss of cytoplasm during enucleation, this procedure was later improved by using a DNA vital stain (Bisbenzimide; Hoechst) and ultraviolet (UV) irradiation to check whether the MII plate after removal of small portions of cytoplasm. The most common procedure of oocyte enucleation is to expose secondary oocytes to bisbenzimide, blindly remove a cytoplasmic fragment surrounding the first polarbody and then expose the oocyte to UV to ascertain whether enucleation was correctly performed. On average this procedure correctly enucleates between 60 to 80 percent of oocytes. Another possible limitation of this procedure is that oocytes are exposed both to UV irradiation and Hoechst 33342 that have been shown to have detrimental effects on the cytoplasm (Smith,L. 1993 *J. Reprod. Fert.* **99**:39-44).

As mentioned above, host oocytes are able to support better development after nuclear transfer when compared to pronuclear-enucleated host zygotesIt has already been shown that MII-stage enucleated oocytes either aged or activated before fusion support better development. The problem of using young non-activated oocytes is caused by incompatibilities between the cell cycle stages of the nuclear donor cell and the host cytoplasm. Metaphase arrested secondary (MII) oocytes have high levels of a Maturation Promoting Factor (MPF), a cellular activity that is responsible for maintaining the chromatin condensed without a nuclear envelop. When blastomere interphase-stage nuclei

containing decondensed chromatin are introduced into an MII oocyte, MPF leads to a rapid breakdown of the nuclear membrane and premature chromosome condensation (PCC). However, PCC is believed to be detrimental only when induced during the DNA synthesis stage (S-phase) of cell cycle. This is particularly problematic when using donor nuclei from blastomeres since these undergo S-phase for most time in between cell divisions. On the other hand, enucleated oocytes that have been activated or aged before fusion to nuclear donor cells have lower levels of MPF and, therefore, do not cause PCC.

With the exception of blastomeres, most other cell types have longer gaps both before (G1-phase) and after (G2-phase) the S-phase and, therefore, are less susceptible to the harmful effects of S-phase PCC when fused to a MII oocytes. Because high MPF levels cause the breakdown of the nuclear membrane, MII stage host oocytes are believed to facilitate interactions between donor nuclei and putative oocyte cytoplasmic 'factors' required for reprogramming the chromatin of nuclei derived from cells further advanced in differentiation. Several examples in the literature report on the advantages of passaging further differentiated donor nuclei in non-activated MII oocytes before activating the reconstructed oocyte. In cattle, nuclei from an embryonic cell line supported significantly higher yield of blastocyst development and more 30d pregnancies when fused to enucleated oocytes 4 h before activation. In mice, significantly more embryos reconstructed with cumulus cell nuclei developed to the blastocyst stage by exposing the donor nucleus to MII cytoplasm for between 1 and 6 h before activation (Wells et al. 1999, *Biol. Reprod.* **60**:996-1005). Moreover, no fetal development or live offspring was obtained when using with simultaneous activation and

fusion. Furthermore, other reports using differentiated cell lines have used host oocytes that were either activated after or concurrently with introducing the donor nucleus (Cibelli et al. 1998, *Nature Biotechnol.* **16**:642-646; Wilmut et al. 1997, *Nature* **385**:810-813). Therefore, the prevalent theory in the field of cloning by nuclear transfer is that a period of reprogramming in the cytoplasm of an inactivated oocyte is required to obtain success when using donor nuclei from cells other than embryonic blastomeres.

It would be highly desirable to be provided with an improved method for obtaining an enucleated host oocyte for transferring nuclei from embryonic, germinal and somatic cells with the objective of cloning or multiplying mammals.

It would be highly desirable to be provided with an improved method of reconstituting an animal embryo.

SUMMARY OF THE INVENTION

The present invention described below is contrary to current knowledge in that we are teaching use of an activated oocyte as recipient for nuclei derived from cells from embryonic and somatic cell lines.

One aim of the present invention is to provide an improved method for obtaining an enucleated host oocyte for transferring nuclei from embryonic, germinal and somatic cells with the objective of cloning or multiplying mammals.

Another aim of the present invention is to provide an improved method of reconstituting a non-human embryo.

In accordance with the present invention there is provided a method of preparing an enucleated host

oocyte for transferring nuclei from embryonic, germinal or somatic cells, which comprises the steps of:

a) activating oocyte by artificial means; and

b) enucleating the activated oocyte when the activated oocyte is undergoing the expulsion of a second polarbody or when the activated oocyte has recently expelled second polarbody (Tel-II); and

c) transferring nuclei from embryonic, germinal or somatic cells into the enucleated oocyte of step b), wherein embryonic cells are cultured prior to nuclei transfer.

The germinal or somatic cells are cultured prior to nuclei transfer.

The oocyte of step a) has a first polarbody and the artificial means is chemical means, such as ethanol or ionomycin.

Step b) may be performed after oocytes are cultured for a period of time sufficient to allow for extrusion of a second polarbody.

Step b) may be performed with oocytes in a medium with cytosqueletal inhibitors.

Step b) may be effected by microsurgically removing the second polar with about one tenth of the cytoplasm surrounding the second polarbody.

The preferred oocyte is a secondary (M-II) oocyte.

In accordance with the present invention, there is provided a method of reconstituting a non-human embryo, which comprises the steps of:

a) activating oocyte by artificial means;

b) enucleating the activated oocyte when the activated oocyte is undergoing the expulsion of a second polarbody or when the activated oocyte

has recently expelled second polarbody (Tel-II);

c) transferring a diploid nucleus in the enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

d) culturing *in vitro* the reconstructed oocyte and/or transferring the reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

In accordance with the present invention, there is provided a method for production of a transgenic non-human embryo, which comprises the steps of:

a) transfecting cultured cells selected from the group consisting of embryonic, germinal and somatic cells with a desired DNA construct;

b) activating oocyte by artificial means;

c) enucleating the activated oocyte when the activated oocyte is undergoing the expulsion of a second polarbody or when the activated oocyte has recently expelled second polarbody (Tel-II);

d) transferring a diploid nucleus extracted from the transfected cells of step a) in the enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* the reconstructed oocyte and/or transferring the reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

The non-human embryo may develop into a non-human animal.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 illustrates a schematic protocol of the technique of telophase enucleation for nuclear transfer.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of producing embryos by nuclear transplantation from embryonic, germinal and somatic cells lines. Nuclear transfer procedures have invariably initiated with the enucleation of host oocyte. The enucleation procedure is followed by one of the following: (a) activation followed by fusion; (b) concurrent activation and fusion; or (c) fusion followed by activation. Whereas the procedure in which oocytes are (a) enucleated, activated and then fused is used mostly for embryonic blastomeres, most techniques applied for further differentiated donor nuclei use the procedure where oocytes are enucleated, (b) fused and activated concurrently or (c) fused and later activated. Although the different approaches in the nuclear transfer procedure have been described previously (U.S. Patent No. 4,994,384; U.S. Patent No. 5,057,420; U.S. Patent No. 5,843,754 and International Patent applications Nos. PCT/GB96/02098, PCT/US98/00002, PCT/US98/12800, PCT/US98/12806, and PCT/US97/12919), the present invention describes a sequence of steps in the nuclear transfer procedure that is novel (Fig. 1).

As illustrated in Fig. 1, Step 1 involves the activation of secondary (M-II) oocytes by artificial means. Step 2 is performed shortly after activation when the oocyte is undergoing the expulsion or recently expelled the second polarbody (Tel-II). Step 3 relates to the transfer of a nucleus from any source with the

purpose of reconstructing the oocyte with a diploid chromosomal content.

Step 1 (oocyte activation)

Oocytes are obtained either *in vivo* or *in vitro* and cultured in maturation medium. After maturation, oocytes are denuded of cumulus cells and those with a first polarbody are parthenogenetically activated by chemical means using ethanol or ionomycin. After activation, oocytes are cultured for a few hours to allow for extrusion of the second polarbody.

Step 2 (oocyte enucleation)

After activation, oocytes can be placed in medium with cytosqueletal inhibitors to facilitate microsurgery. Only oocytes with a second polarbody extruded or partially extruded are used. Approximately one tenth of the cytoplasm surrounding the second polar body is microsurgically removed with the second polarbody.

Step 3 (nuclear transfer)

After enucleation, a single cell containing a diploid nucleus is introduced into the enucleated oocyte either by cell fusion or microinjection (nuclear transfer). The reconstructed oocyte is then cultured *in vitro* and/or transferred into the reproductive tract of a suitable surrogate mother to enable further development.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE 1

Telophase Enucleation

Follicles with 2 to 8 mm diameter were aspirated from bovine slaughterhouse ovaries. Oocytes with a homogeneous cytoplasm and several layers of cumulus cells were selected and placed in maturation within 1 h from follicular aspiration. At 28 h after maturation oocytes were denuded of cumulus cells and those with a first polarbody were used in the experiment. Oocytes were exposed to 7% ethanol for 5 min, washed and placed in maturation medium for different periods. At 1 h before microsurgery, oocytes were placed in cytochalasin B and positioned for micromanipulation. Oocytes undergoing extrusion or already with extruded second polarbodies had 10% of their cytoplasmic volume removed together with the second polarbody. After microsurgery, oocytes were fixed in 10% formalin, stained with 5 µg Hoechst 33342 and observed under UV epi-fluorescence. Oocytes without any chromatin were considered successfully enucleated. Most oocytes were successfully enucleated when micromanipulated at the times examined (Table 1). Because the efficiency of this enucleation technique is high, checking of oocytes with DNA stain and UV light is not necessary. Significantly lower percentages of enucleation was obtained when blindly removing using the position of the first polarbody to aspirate 30% of the surrounding cytoplasm in oocytes at metaphase (59%) at 24 h from the beginning of *in vitro* maturation.

Table 1

Successful telophase enucleations as performed at different times after exposure to a stimulus to parthenogenetically activate secondary oocytes

	Time after activation			
	3 h	4 h	5 h	Total
Number manipulated	37	38	43	118
Successful enucleation (%)	36 (97%)	37 (97%)	40 (93%)	113 (96%)

Example 2

Nuclear transfer with morula-stage blatomeres

Bovine secondary oocytes were matured *in vitro* and enucleated using the technique described above (telophase enucleation). Morula-stage embryos were disaggregated and individual blastomeres were inserted into the perivitelline space of enucleated oocytes. Fusion between the membranes of blastomeres and oocytes was obtained with an electric pulse that causes fusion between the membranes of the donor and recipient cells. The electrical parameters used were double 60 µsec pulses of 1.5 KVolts per cm. After fusion the embryos were cultured for 7 days in the presence of Menezo's B2 medium supplemented with 10% fetal calf serum.

Table 2

Fusion and development of bovine oocytes reconstructed with nuclei from morula-stage blastomeres recovered 5 days after IVF

	Number	Fused	Blastocyst	No. nuclei
Telophase II (%)	215	129 (58%)	49 (38%)	126±11
Metaphase II (%)	248	151 (60%)	24 (16%)	84±9

Example 3

Nuclear transfer with non-starved bovine ES cells

Bovine embryo stem (ES)-like cells were obtained from day 8 blastocyst stage embryos produced entirely *in vitro*. ICMs were platted onto mitomycin-inactivated mouse fibroblasts. Established ES-like lines were disaggregated by short exposure to trypsin using a narrow pipette. Isolated cells were placed in the perivitelline space of enucleated oocytes and exposed to an electric pulse that causes fusion between the membranes of the donor and recipient cells. The electrical parameters used were double 100 µsec pulses of 1.5 KVolts per cm. Electrical stimulation was performed as soon as possible after placing the nuclear donor cell in the perivitelline space to obtain better fusion results. After fusion the embryos are cultured for 7 days in the presence of Menezo's B2 medium supplemented with 10% fetal calf serum.

Table 3

Fusion and development of bovine oocytes reconstructed with nuclei from ES-like cells exposed to 5% of FCS

	Number	Fused	Cleaved	Blastocyst
Telophase II	38	11	5	3
(%)		(30%)	(45%)	(27%)
Metaphase II	33	12	2	1
(%)		(36%)	(17%)	(8%)

Example 4

Nuclear transfer with serum-starved bovine ES cells

Bovine embryo stem (ES)-like cells were cultured in medium with 0.5% FCS for 5 days before micromanipulation. As described above, ES-like cells were disaggregated, placed in the perivitelline space of enucleated oocytes and exposed to an electric pulse to cause fusion between the membranes of the donor and

recipient cells. After fusion the embryos are cultured for 7 days in the presence of Menezo's B2 medium supplemented with 10% fetal calf serum.

Table 4

Fusion and development of bovine reconstructed with nuclei from bovine ES-like cells exposed (starved) to low concentrations (0.5%) of FCS

	Number	Fused	Cleaved	Blastocyst
Telophase II(%)	38	13(34%)	3(23%)	2(27%)
Metaphase II(%)	42	13(31%)	4(31%)	1(15%)

Example 5

Nuclear transfer with starved and non-starved bovine fetal fibroblasts

Bovine fetal fibroblast cells were recovered form day 50 fetuses and passaged in medium D-MEM with 10% FCS. Non-starved fibroblast cells were recovered during growth at 2 days after passaging. Serum starved cells were exposed to medium with 0.5% serum for 5 days before NT. NT was performed as described above.

Table 5

Fusion and development of bovine reconstructed with nuclei from bovine fetal fibroblast cells exposed for 5 days to low concentrations (0.5%) of FCS (starved) or to 5% FCS for 20 h after seeding (non-starved)

	Serum starved			Non-starved		
	Number	Fused	Blast.	Number	Fused	Blast.
Telophase II	69	52	2	105	67	9
(%)		(75%)	(4%)		(64%)	(13%)
Metaphase II	60	39	9	114	92	12
(%)		(65%)	(24%)		(81%)	(13%)

Example 6
Nuclear transfer with starved and non-starved bovine fetal fibroblasts transfected with a GFP construct

Bovine fetal fibroblast cells were recovered form day 50 fetuses and passaged in medium D-MEM with 10% FCS. The fetal fibroblast cells were transfected with a constructs containing the CMV/eGFP gene (plasmid pGREEN LANTERN-1, Life Technologies). This construct contains the reporter gene Green Fluorescence Protein (GFP) from Aequorea victoria jellyfish, which codes for a naturally fluorescent protein requiring no substrate for visualization. The GFP used is "humanized" (ie., codon sequence) and mutated to contain threonine at position 65 to enhance fluorescence peaking. The advantage of using this fluorescent gene as a reporter being that it yields bright green fluorescence when living or fixed cells are illuminated with blue light and increases our sensitivity of detection. The plasmid contains the CMV immediate early enhancer/promoter upstream of the GFP gene, followed by SV40 t-intron and polyadenylation signal. NT was performed as described above.

Table 6

Fusion and development of bovine reconstructed with nuclei from bovine fetal fibroblast cells transfected with a GFP construct and starved for 4 days and transferred to metaphase stage-enucleated oocytes or cultured for 6 h after thawing and transferred to telophase stage-enucleated oocytes

	Number	Fused	Blastocyst
Telophase II(%)	187	131(71%)	15(11%)
Metaphase II(%)	209	169(81%)	23(14%)

Table 7

Post-implantation development of cloned blastocyts derived from GFP-positive fetal fibroblasts (Table 6)

	No Embryos	No Recipients	Non-returned	60 d positive	200 d positive	liveborn
Telophase II(%)	11	6	2	1	1	1
Metaphase II(%)	15		5	4	4	3

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of preparing a reconstructed non-human oocyte by transferring cell or nucleus from germinal or somatic cells into an enucleated host oocyte, which comprises the steps of:

a) activating said host oocyte;

b) enucleating said activated host oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has expelled said second polarbody (Tel-II); and

c) transferring nucleus from germinal or somatic cells into said enucleated host oocyte of step b) to obtain a reconstructed oocyte.

2. The method according to claim 1, wherein said transferred cell or nucleus is at nuclear stage G0, G1, S, G2, or M.

3. The method of claim 1, wherein said germinal or somatic cells of step c) are cultured prior to nucleus transfer.

4. The method of claim 1, wherein said oocyte of step a) is a secondary oocyte (M-II) and said activation is performed by artificial means selected from the group consisting of physical means and chemical means.

5. The method of claim 4, wherein said chemical means is ethanol or ionomycin.

6. The method of claim 4, wherein said physical means is selected from the group consisting of

electrical means, thermal means, and irradiation technology.

7. The method of claim 1, wherein step b) is performed after oocytes are cultured for a period of time sufficient to allow for at least partial extrusion of a second polarbody.

8. The method of claim 1, wherein step b) is performed with oocytes in a medium with cytosqueletal inhibitors.

9. The method of claim 7, wherein step b) is effected by microsurgically removing said second polarbody with a portion of the cytoplasm containing chromosomes surrounding said at least partially extruded second polarbody.

10. A method of reconstituting a non-human embryo, which comprises the steps of:

a) activating oocyte by artificial or natural means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) culturing germinal or somatic cell prior to nucleus transfer;

d) transferring a nucleus from said cell of step c) in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed

oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

11. The method according to claim 10, wherein said transferred cell or nucleus is at nuclear stage G0, G1, S, G2, or M.

12. The method of claim 10, wherein said oocyte of step a) is a secondary oocyte (M-II) and said artificial means is physical or chemical means.

13. The method of claim 12, wherein said chemical means is ethanol or ionomycin.

14. The method of claim 12, wherein said physical means is selected from the group consisting of electrical means, thermal means, and irradiation technology.

15. The method of claim 13, wherein step b) is performed after oocytes are cultured for a period of time sufficient to allow for at least partial extrusion of a second polarbody.

16. The method of claim 15, wherein step b) is performed with oocytes in a medium with cytosqueletal inhibitors.

17. The method of claim 15, wherein step b) is effected by microsurgically removing said second polarbody with a portion of the cytoplasm containing chromosomes surrounding said at least partially extruded second polarbody.

18. The method of claim 17, wherein step c) is effected by introducing a single cell containing a diploid nucleus into said enucleated oocyte by cell fusion or by microinjection.

19. The method of claim 10, wherein said non-human embryo develops into a non-human animal.

20. A method for production of a transgenic non-human embryo, which comprises the steps of:

a) activating oocyte by artificial or natural means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) culturing germinal or somatic cell prior to nucleus transfer;

d) transferring a transgenic nucleus from said cell of step c) transfected with a desired DNA construct in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

21. The method according to claim 20, wherein said transferred cell or nucleus is at nuclear stage G0, G1, S, G2, or M.

22. The method according to claim 20, which further comprises developing said non-human embryo into a fetus.

23. The method according to claim 22, which further comprises developing said fetus into an offspring.

24. The method of claim 20, wherein said non-human embryo develops into a non-human animal.

25. A transgenic embryo obtained according to the method which comprises the steps of:

a) activating oocyte by artificial or natural means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) culturing germinal or somatic cell prior to nucleus transfer;

d) transferring a transgenic nucleus from said cell of step c) transfected with a desired DNA construct in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

26. A transgenic fetus obtained according to the method which comprises the steps of:

a) activating oocyte by artificial or natural means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) culturing germinal or somatic cell prior to nucleus transfer;

d) transferring a transgenic nucleus from said cell of step c) transfected with a desired DNA construct in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

27. A transgenic offspring obtained according to the method which comprises the steps of:

a) activating oocyte by artificial or natural means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) culturing germinal or somatic cell prior to nucleus transfer;

d) transferring a transgenic nucleus from said cell of step c) transfected with a desired DNA construct in said enucleated oocyte to

obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo..

28. A method of cloning a non-human animal by cell or nuclear transfer which comprises the steps of :

a) activating oocyte by artificial means;

b) enucleating said activated oocyte when said activated oocyte is undergoing the expulsion of a second polarbody or when said activated oocyte has recently expelled second polarbody (Tel-II);

c) culturing germinal or somatic cell prior to nucleus transfer;

d) transferring a diploid nucleus from said cell of step c) in said enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* said reconstructed oocyte and/or transferring said reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

29. The method according to claim 28, wherein said transferred cell or nucleus is at nuclear stage G0, G1, S, G2, or M.

30. The method of claim 28, wherein said oocyte of step a) is a secondary oocyte (M-II) and said artificial means is physical or chemical means.

31. The method of claim 30, wherein said chemical means is ethanol or ionomycin.

32. The method of claim 30, wherein said physical means is selected from the group consisting of electrical means, thermal means, and irradiation technology.

33. The method of claim 28, wherein step b) is performed after oocytes are cultured for a period of time sufficient to allow for at least partial extrusion of a second polarbody.

34. The method of claim 30, wherein step b) is performed with oocytes in a medium with cytosqueletal inhibitors.

35. The method of claim 31, wherein step b) is effected by microsurgically removing said second polarbody with a portion of the cytoplasm containing chromosomes surrounding said at least partially extruded second polarbody.

36. The method of claim 32, wherein step c) is effected by introducing a single cell containing a diploid nucleus into said enucleated oocyte by cell fusion or by microinjection.

37. The method of claim 28, wherein said nucleus or cell of step c) is transgenic or non-transgenic.

38. The method of claim 28, wherein said non-human embryo develops into a non-human animal.

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

Declaration Submitted with Initial Filing OR ☒ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	1051-1-020
First Named Inventor	Lawrence C. Smith
COMPLETE IF KNOWN	
Application Number	10 / 019,375
Filing Date	October 26, 2001
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TELOPHASE ENUCLEATED OOCYTES FOR NUCLEAR TRANSFER

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) April 27, 2000 as United States Application Number or PCT International Application Number PCT/CA00/00483 and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)
60/131,469	April 28, 1999

☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

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DECLARATION — Utility or Design Patent Application

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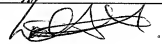
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR : [ ] A petition has been filed for this unsigned inventor

Given Name (first and middle [if any]): 1-00 Lawrence C. | Family Name or Surname: SMITH

Inventor's Signature:  | Date: November 13, 2001

Residence: City Saint-Hyacinthe | State: Quebec | Country: Canada | Citizenship: Canada CAN

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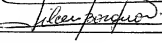
NAME OF SECOND INVENTOR: [ ] A petition has been filed for this unsigned inventor

Given Name (first and middle [if any]): 2-00 Vilceu | Family Name or Surname: BORDIGNON

Inventor's Signature:  | Date: November 13, 2001

Residence: City Saint-Hyacinthe | State: Quebec | Country: Canada | Citizenship: Canada CAN

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City: Saint-Hyacinthe | State: Quebec | ZIP: J2S 7H8 | Country: Canada

[ ] Additional inventors are being named on the ____supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.

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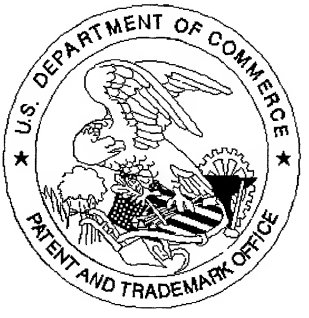

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